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Report

Circadian Regulation in the Ability of *Drosophila* to Combat Pathogenic Infections

Jung-Eun Lee[1] and Isaac Edery[1,2,*]
[1]Graduate Program in Biochemistry and
[2]Department of Molecular Biology and Biochemistry
Center for Advanced Biotechnology and Medicine
Rutgers University
679 Hoes Lane
Piscataway, New Jersey 08854

Summary

We sought to determine if the innate immune response is under circadian regulation and whether this impacts overall health status. To this end, we used infection of *Drosophila* with the human opportunistic pathogenic bacteria *Pseudomonas aeruginosa* as our model system [1]. We show that the survival rates of wild-type flies vary as a function of when, during the day, they are infected, peaking in the middle of the night. Although this rhythm is abolished in clock mutant flies, those with an inactive *period* gene are highly susceptible to infection, whereas mutants with impairment in other core clock genes exhibit enhanced survival. After an initial phase of strong suppression, the kinetics of bacterial growth correlate highly with time of day and clock mutant effects on survival. Expression profiling revealed that nighttime infection leads to a clock-regulated transient burst in the expression of a limited number of innate immunity genes. Circadian modulation of survival also was observed with another pathogen, *Staphylococcus aureus*. Our findings suggest that medical intervention strategies incorporating chronobiological considerations could enhance the innate immune response, boosting the efficacy of combating pathogenic infections.

Results and Discussion

Time of Day and Clock Mutant Effects on the Survival Rates of *Drosophila* Infected with *P. aeruginosa*

The innate immune response [2, 3] and circadian clock mechanisms [4] are both highly conserved between *Drosophila* and mammals. *P. aeruginosa* is a human opportunistic pathogen commonly found in hospital-acquired infections [1], and studies that use *Drosophila* have revealed numerous insights into understanding the pathogenicity of these Gram-negative bacteria [5]. To test if the circadian system modulates the ability of *D. melanogaster* to combat a pathogenic infection, we first entrained control rhythmic flies (*yw*) under standard 12 hr light/12 hr dark cycles (LD; where zeitgeber time 0 [ZT0] is defined as lights on) for 2 days. On the third day, we inoculated them at different times of day with the PA14-isogenic mutant strain of *P. aeruginosa*, which is defective in *phopholipase C* (PA14 *plcs*) [6] (Figures 1A and 1B and Figure S1 available online). The PA14 *plcs* strain is a less virulent one than PA14 and was chosen in our studies because although infection with this attenuated strain evokes rapid mortality (between 1–2 days) of many flies, a sizable proportion survives throughout an extended postinfection observation period (at least 1 week in our standard experimental setup; termed "survivors") (Figures 1A and 1B), as previously reported [6]. By establishing conditions that yielded a mixed population response with individuals that succumbed quickly to the infection and those that survived over an extended period of time, this allowed us to better evaluate whether the clock modulates the ability of flies to successfully combat a pathogenic infection. Adult flies were infected by the standard method of lightly stabbing their abdomens with a fine needle dipped in a concentrated liquid culture containing PA14 *plcs*. We also included control groups that were contemporaneously mock treated with needles placed in just the growth media (on average, 90%–100% of the flies stabbed with control needles survived to the end of the test period; Table S1).

Control (*yw*) flies exhibit a diurnal profile in their survival rates (one-way ANOVA, $p < 0.0005$ for Figures 1A and 1B). Tukey-Kramer Honestly Significant Difference (HSD) analysis indicated that control flies infected at ZT21 have significantly higher rates of survival than those infected at ZT1, ZT5, or ZT9 (when $\alpha = 0.01$; see also the legend to Figure 1). Flies infected at ZT21 survived approximately 4-fold better compared to the trough values at ZT5 (two-tailed Student's t test, $p < 0.005$ at 48 hr postinfection and thereafter) (Figures 1A and 1B). We observed a similar daily pattern in survival rates when inoculating flies with bacterial titers 5- to 20-fold lower compared to our standard conditions (compare Figures 1A and 1B to Figure S1), demonstrating that the time-of-day effects on survival are observed over a broad range of initial bacterial doses.

To determine if the survival rhythm is endogenously driven, flies were entrained by three LD cycles and subsequently maintained in constant darkness (DD) followed by inoculation on the second day of DD. In addition, we also infected the well-characterized per^{01}, tim^{01}, Clk^{Jrk}, and cyc^{01} arrhythmic clock mutants that carry inactivated *period* (*per*), *timeless* (*tim*), *clock* (*Clk*), and *cycle* (*cyc*) genes, respectively [4]. To minimize genetic background effects, the clock mutants were evaluated in the same *yw* background as the control strain.

Daily changes in the survival rates of control rhythmic flies were also observed in DD (Figure 1B; one-way ANOVA, $p < 0.005$; statistical analysis summarized in the legend to Figure 1 and in Table S2). The profile in constant darkness was almost identical to that observed in LD except that flies infected at circadian time 17 (CT17; in this manuscript we use the term CT as equivalent to ZT, which is a reasonable approximation given the near 24 hr behavioral rhythms of *Drosophila*) showed the best survival rates (Tukey-Kramer HSD when $\alpha = 0.01$). Flies infected at CT17 survived approximately 3-fold better compared to the trough values observed at CT5 (two-tailed Student's t test, $p < 0.005$ at 48 hr postinfection and thereafter) (Figures 1A and 1B and Table S2). Importantly, similar results whereby survival rates are significantly higher at CT17 compared to CT5 also were observed when using rhythmic flies with different genetic backgrounds, including the *Canton-S* (CS) and *Oregon R* (OR) wild-type strains (Figure 2A; two-tailed Student's t test, $p < 0.05$, and data not shown).

*Correspondence: edery@cabm.rutgers.edu

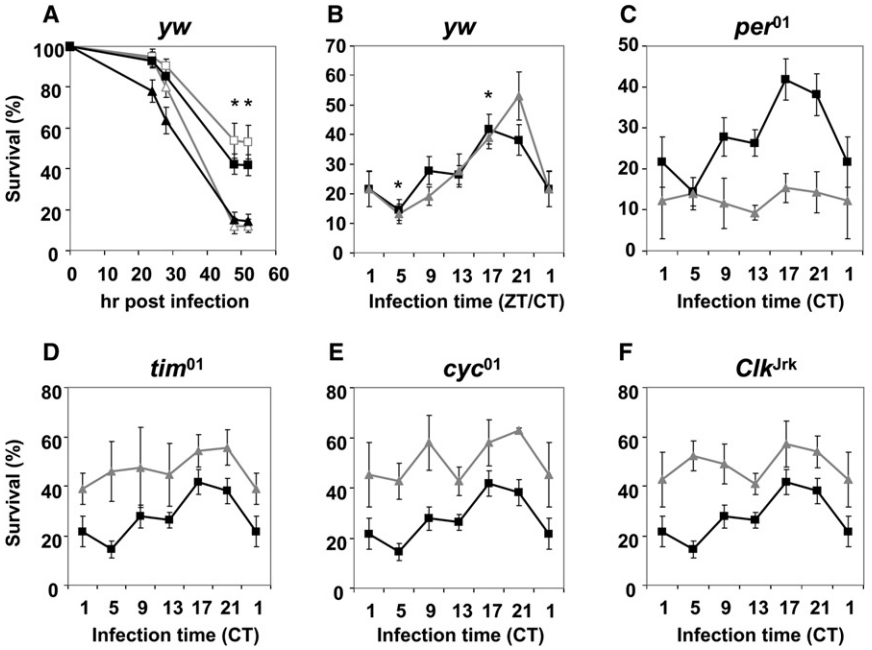

Figure 1. Time of Infection during a Daily Cycle and Mutations in Clock Genes Modulate the Survival Outcomes of Flies Infected with *P. aeruginosa*

(A) Time course in the proportion of *yw* flies surviving after infection with PA14 *plcs* at ZT5 (open triangles, n = 251; indicates total number of flies from several independent experiments that were infected and used to calculate the average survival data shown) and ZT21 (open squares, n = 249) during LD, or CT5 (filled triangles, n = 321) and CT17 (filled squares, n = 321) during DD. Asterisks (*) indicate significantly higher survival rates for the ZT21 or CT17 groups compared to the ZT/CT5 groups (two-tailed Student's t test; *p < 0.005).

(B) Survival rates of *yw* flies infected with PA14 *plcs* at different times of day during either LD (gray triangles, n = 246–253; i.e., indicates the range in the total number of flies from several independent experiments that were infected at the different times in a daily cycle) or DD (black squares, n = 315–323). Survival profiles were evaluated by one-way ANOVA followed by Tukey-Kramer HSD analysis with the following results. (1) In LD, the ZT21 group survived better than did the ZT1, ZT5, or ZT9 groups (one-way ANOVA, p < 0.0005; Tukey-Kramer HSD, $\alpha = 0.01$). At $\alpha = 0.001$, only the ZT5 group died significantly more than the ZT21 group. (2) In DD, the CT17 and CT21 groups have higher survival rates compared to the CT5 group (one-way ANOVA, p < 0.005; Tukey-Kramer HSD, $\alpha = 0.05$). At $\alpha = 0.01$, only the CT17 group exhibited significantly higher survival compared to the CT5 group (*).

(C–F) Survival rates of clock mutant flies (gray triangles) infected with PA14 *plcs* during DD compared to control *yw* flies (black squares). Results reflect the average of at least three independent experiments. Error bars indicate standard error of the mean (SEM).

In contrast to rhythmic control and wild-type strains, a daily survival rhythm was not observed in the four arrhythmic clock mutant strains tested (Figures 1C–1F; one-way ANOVA, p > 0.05; Table S2). More extensive analysis comparing CT5 and CT17 groups did not reveal significant time-of-day differences in the survival rates of per^{01} or Clk^{Jrk} flies (Table S3). Although we cannot rule out the possibility of small but real time-of-day variations in the survival rates of the clock mutants (Tables S2 and S3), our data indicate that the robust daily rhythm observed in control and wild-type flies is either abolished or greatly attenuated in the mutants. In addition, close inspection of the survival patterns of the clock mutants suggests the possibility of low-amplitude cycles that peak twice per day (Figures 1C–1F). Intriguingly, in many cases, higher frequency "ultradian" rhythms are more readily observed or enhanced when circadian systems are severely compromised [7].

Although we do not observe robust daily rhythms in survival for the different arrhythmic clock mutants analyzed, per^{01} flies manifested relatively higher mortality rates compared to control flies, whereas tim^{01}, Clk^{Jrk}, and cyc^{01} flies showed overall enhanced survival compared to control flies (Figures 1C–1F). Similar results also were obtained when examining the survival patterns of the clock mutants in LD, except that mortality of all the clock mutants tested was overall slightly higher during the daytime, suggesting that in these mutants the light/dark conditions have direct effects on the ability to survive the infection (data not shown). While our manuscript was under review, Shirasu-Hiza et al. (2007) reported that per^{01} flies were more susceptible to *Streptococcus pneumoniae* and *Listeria monocytogenes* compared to wild-type flies [8], consistent with our findings. However, in that study tim^{01} flies also succumbed to death faster than wild-type flies when infected with the same pathogens. The possible discrepancy between the two studies with regard to the ability of tim^{01} flies to survive pathogenic infections is presently unclear and might be due to the use of different bacteria and/or the mode of pathogenicity. Although the reasons underlying the differential effects of clock mutations are not known, the collective findings suggest that *per* function plays a protective role in *Drosophila* infected with pathogenic bacteria. Future studies will be required to better evaluate the roles of the different clock genes in innate immunity.

To expand our observations we also inoculated wild-type flies with another human pathogenic bacteria, *Staphylococcus aureus* (*S. aureus*), which, unlike *P. aeruginosa*, is Gram-positive. As with *P. aeruginosa*, the CT17 group exhibited higher rates of survival compared to the CT5 group (Figure 2B; two-tailed Student's t test, p < 0.05). Taken together, our findings indicate that *Drosophila* survive nighttime infections significantly better than daytime ones.

Bacterial Growth Kinetics Correlate with Survival Rates in Rhythmic and Clock Mutant Flies

To determine whether the kinetics of bacterial growth correlate with the survival patterns observed, we infected control, per^{01} and Clk^{Jrk} flies on the second day of DD at either CT5 or CT17, the trough and peak times for survival rates, respectively (Figure 1). Live flies were collected at several times postinoculation and bacterial titers measured (Figure 3). In each experiment, all three genotypes were contemporaneously treated and the results from multiple experiments pooled. Also, a subset of flies were not processed for the bacterial growth assays but were scored for survival and, in rare cases where anomalous survival results were obtained (e.g., little or no mortality), the bacterial data from that experiment were not used. Irrespective of the infection time, all genotypes showed strong decreases in bacterial titers during the first 5 hr postinfection (Figures 3A–3C). However, in control *yw* flies, the CT5 group

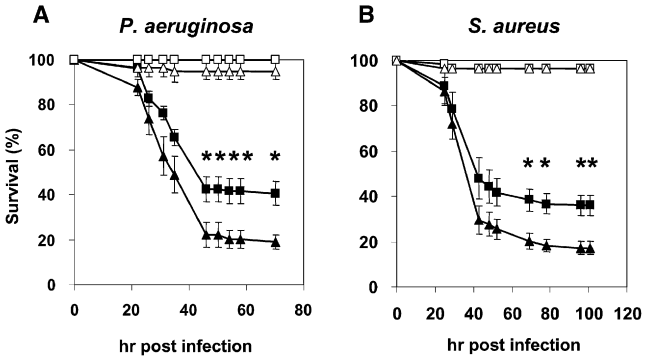

Figure 2. Nighttime Infections Lead to Higher Survival Rates in a Variety of Wild-Type *D. melanogaster* Strains Infected with Either *P. aeruginosa* or *S. aureus*

(A) Time course depicting the survival rates of wild-type flies (*Canton-S* and *Oregon R*) infected with PA14 *plcs* at CT5 (filled triangles) and CT17 (filled squares) on the second day of DD. Also shown are mock-injury groups pricked at CT5 (open triangles) and CT17 (open squares). Very similar survival curves were obtained for *Canton-S* and *Oregon R* flies (data not shown), and hence the data were pooled. Results are the average of four independent experiments and indicate significantly higher survival rates for flies infected at CT17 compared to CT5 (two-tailed Student's t test; CT5 versus CT17, *p < 0.05). Error bars indicate SEM. The number of flies analyzed is as follows: n = 151 for the infected CT5 group, n = 154 for the infected CT17 group, and n = 70 for the mock-injury CT5 or CT17 groups.

(B) Time course depicting the survival rates of wild-type flies (*Canton-S* and *Oregon R*) infected with *S. aureus* at CT5 (filled triangles) and CT17 (filled squares). Also shown are mock-injury groups pricked at CT5 (open triangles) or CT17 (open squares). Results are the average of four independent experiments and indicate significantly higher survival rates for flies infected at CT17 compared to CT5 (two-tailed Student's t test; CT5 versus CT17, * p < 0.05). Error bars indicate SEM. The number of flies analyzed is as follows: n = 160 for the infected CT5 or CT17 group and n = 80 for the mock-injury CT5 or CT17 groups.

had significantly higher bacterial loads at 10 hr and 23 hr postinfection compared to the CT17 group (two-tailed Student's t test, $p < 0.05$ for 10 hr, $p = 0.01$ for 23 hr), whereas per^{01} and Clk^{Jrk} flies did not exhibit significant differences in bacterial loads as a function of infection time (Figures 3A–3D; statistical analysis summarized in Table S4). Nonetheless, the titer of PA14 *plcS* in per^{01} flies increased between 5 hr to 10 hr postinfection but remained very low in Clk^{Jrk} flies (Figures 3B and 3C). Indeed, pairwise comparisons indicated significantly higher levels of bacteria in per^{01} flies at 10 hr and 23 hr postinfection compared to Clk^{Jrk} flies (Table S4), consistent with their relatively lower survival rates (Figure 1 and Figure S2).

To further demonstrate that per^{01} flies have higher mortality rates compared to Clk^{Jrk} flies, we infected per^{01} flies with approximately half the number of bacteria used to infect Clk^{Jrk} flies (Figure S2A) and compared their survival rates (Figure S2B). Despite the lower bacterial load used in the infection, significantly more per^{01} flies died compared to Clk^{Jrk} flies (two-tailed Student's t test, $p < 0.01$ after 52 hr post infection, $\alpha = 0.05$; Figure S2B). These results are consistent with the observation that similar survival rhythms are observed in control flies over a wide range of initial bacterial doses (Figure S1). Thus, the time-of-day differences observed in rhythmic flies and the enhanced survival of Clk^{Jrk} compared to per^{01} flies cannot be accounted for by possible experimental variations in the amount of bacteria used during inoculation.

In summary, after an initial phase of bacterial clearance, there is a tight correlation between bacterial loads and survival outcomes, both for wild-type flies as a function of circadian time and when comparing clock mutants. Indeed, the early bacterial growth pattern in per^{01} flies mimics that observed in the wild-type CT5 group, increasing after 5 hr postinfection, whereas the Clk^{Jrk} response is more similar to the wild-type CT17 group, which declines or remains low at 10 hr postinfection. Thus, our findings suggest that the ability to suppress bacterial growth during the first 10 hr after infection is linked causally to better prognosis for survival.

Clock Regulation in the Induced Profiles of Innate Immunity Genes Is Highly Selective and Restricted to the Early Phase of the Infection

The best-studied defense effectors in innate immunity are antimicrobial peptide genes (AMPs) that are rapidly induced after microbial infection [2]. In *Drosophila*, AMPs are primarily induced via activation of the Toll (mainly responding to fungi or Gram-positive bacteria) and/or Imd (mainly responding to Gram-negative bacteria) pathways [2]. As an initial attempt to understand the molecular mechanisms underlying the circadian pattern of survival rates and bacterial growth kinetics, we focused largely on the expression patterns of several key players in the Toll and Imd innate immune signaling pathways activated by microbial infection. This included measuring the postinoculation expression kinetics of several peptidoglycan recognition proteins (PGRP) shown to play central roles as microbial receptors and/or scavengers (e.g., PGRP-SA, -LC and -LB), AMPs (i.e., *attacin* A [*attA*], *defensin* [*def*], *diptericin* [*dipt*], *drosocin* [*drc*], and *drosomycin* [*drs*]), and some key signaling components such as *imd*. Control *yw*, per^{01}, and Clk^{Jrk} flies were infected during the second day of DD and collected at different times postinfection. We used real-time quantitative RT-PCR to measure RNA levels in head extracts of adult flies because we noted that the induced levels of many immune relevant genes attain higher values in heads compared to isolated bodies or whole flies (e.g., compare Figure 4 and Figure S4; data not shown). Expression of immune genes in the head has been described elsewhere [9] (data not shown). For each genotype and gene surveyed, we compared the RNA values obtained at the same postinfection time point for the CT5 and CT17 infected groups.

Intriguingly, although many immune relevant genes are induced after infection with *Pseudomonas* [10], the postinoculation expression patterns of only *PGRP-SA* and *drc* showed differences as a function of infection time in control flies, which were abolished in the clock mutants, indicative of bona fide circadian regulation (Figure 4 and Figure S3). For *PGRP-SA*, its mRNA levels at 2 hr and 5 hr postinfection are significantly higher in the CT17 group compared to the values obtained at the same postinfection time points in the CT5 group (Figure 4A), whereas for *drc* higher mRNA levels were observed at 5 hr postinfection in the CT17 group compared to the same postinfection time point in the CT5 group (Figure 4D). Similar circadian patterns in the induction profiles for *PGRP-SA* and *drc* were also obtained when analyzing extracts prepared from isolated bodies (Figure S4).

Besides *imd*, none of the immune relevant genes we probed exhibit circadian fluctuations in basal levels (data not shown; e.g., endogenous levels of *imd* are higher at CT17 compared to CT5 in control flies, Figure S3M, compare values at 0 hr postinfection). This is consistent with prior work that uses microarrays to probe daily patterns of gene expression in head extracts [11]. However, we did not observe time-of-day differences in the induced levels of *imd* after infection (Figure S3M). Thus, whether the basal expression of an immune response

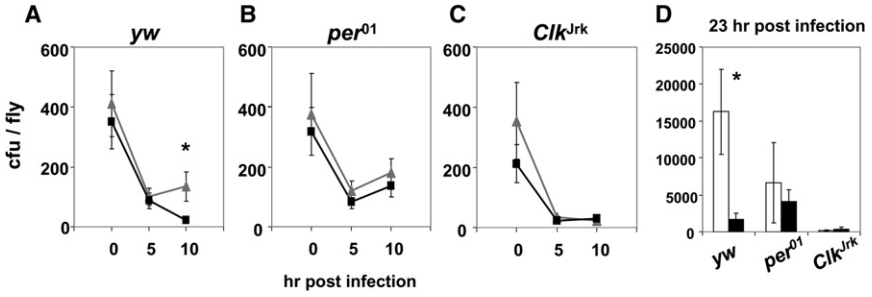

Figure 3. Bacterial Growth Correlates with Time of Infection and Clock Mutant Effects on Survival Rates

(A–D) Flies of the indicated genotype were infected at either CT5 (gray triangles or open bars) or CT17 (black rectangles or filled bars) and collected at the indicated times. Asterisks (*) indicate significantly higher bacterial titer for the control CT5 group compared to the CT17 group (two-tailed Student's t test, $p < 0.05$). Results from at least three independent experiments were combined. Mean ± SEM and values obtained from at least 60 flies per collection time are displayed (n = 60–128, n = 95–130, and n = 67–90 for *yw*, *per*^{01}, and *Clk*^{Jrk} flies, respectively).

gene is constitutive or circadian is not necessarily linked to how the clock regulates its expression postinfection. Although it is not clear why the induced profile of *drc* and not other AMP genes exhibits circadian differences as a function of infection time (compare Figure 4 and Figure S3), Drosocin kills Gram-negative bacteria [2] and has been demonstrated to be one of only a few AMPs that when overexpressed can protect flies infected with *P. aeruginosa* [10]. In this context it is interesting to note that although PGRP-SA has a characterized role as a receptor in the Toll pathway [12], it has recently been implicated in phagocytosis as well [13].

Together, our findings suggest the following scenario for how the clock in *Drosophila* might influence the progression of an infection with *P. aeruginosa*. Early during the infection a robust immune response (perhaps both cellular and humoral) is mounted, which is effective in pathogen clearance irrespective of when during a daily cycle the flies are infected, as indicated by the rapid drop in bacterial titer during the first 5 hr postinfection (Figure 3A). However, the clock regulates the induced levels of a limited subset of innate immunity players, such as *PGRP-SA* and *drc*, whereby infections in the middle of the night result in a transient burst early during the infection (Figures 4A and 4D). Higher levels of a few key immune players over a certain threshold may contribute to keeping the titer of pathogenic bacteria low after the initial rapid-declining phase (Figure 3A). By prolonging the suppression of bacterial growth during a critical window of the infection, this might provide an opportunity to mount or recruit additional host defenses in addition to AMPs, resulting in improved survival (Figures 1A and 1B). Thus, our results suggest that the clock modulates the strength or responsiveness of immune activation in a time-of-day-dependent manner but only during a critical early phase of the infection process that has physiological consequences on the ability of the host to survive pathogenic infections. Indeed, it is noteworthy that *P. aeruginosa* eludes host defenses by the early suppression of antimicrobial peptide gene expression [10]. It will be of interest to determine why the postinfection expression profiles of only certain immune response genes exhibit circadian regulation and how this is apparently restricted to a particular phase of the immune response.

Although the time-of-day differences in the levels of induced *PGRP-SA* and *drc* are clearly consistent with the survival rates of wild-type flies infected at different times of day, this is not the case for the *per*^{01} and *Clk*^{Jrk} mutants in which the overall levels of *drc* are much lower in *Clk*^{Jrk} compared to *per*^{01} flies (Figure 4; a trend observed for other AMP genes surveyed, Figure S3 and data not shown). Although seemingly paradoxical, this is not unanticipated as there are precedents in the literature showing that flies can be more susceptible to bacterial infection despite elevated levels of AMP expression, indicating that excessive or inappropriate immune activation can be deleterious [14–17]. In this context it is important to consider that

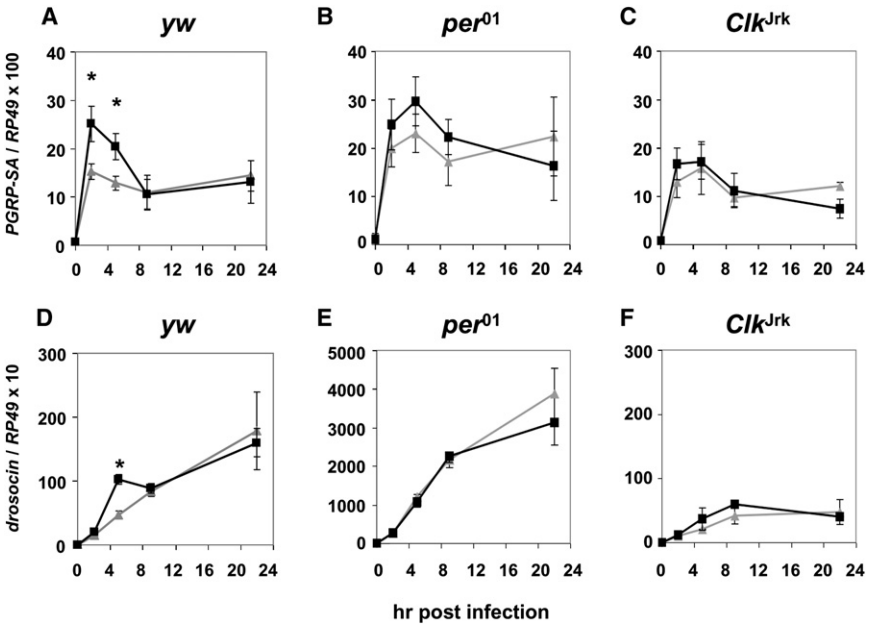

Figure 4. Nighttime Infection Leads to Early and Transient Clock-Regulated Increases in the mRNA Induction Profiles of a Limited Number of Immune Response Genes

(A–F) Flies were infected at either CT5 (gray triangles) or CT17 (black squares), collected at the indicated times, and RNA levels measured. For each genotype we compared the RNA values for the CT5 and CT17 groups that were obtained at the same postinfection time point. Asterisks (*) indicate significantly higher mRNA levels for the *yw* CT17 group compared to the *yw* CT5 group (two-tailed Student's t test, $p < 0.0005$ for *drosocin*, $p < 0.05$ for *PGRP-SA*). Results from at least three independent experiments were averaged except that *Clk*^{Jrk} data were derived from two experiments. Error bars indicate SEM.

besides the production of AMPs, innate immunity in adult *Drosophila* includes a proteolytic cascade leading to melanization and a cellular immune response characterized by phagocytosis [18]. It is possible that inactivation of *per* might affect other host defense mechanisms that cannot be compensated by a potentially hypersensitive humoral immune response. Conversely, Clk^{Jrk} and other clock mutant flies (Figure 1) might have a heightened activity of cellular immunity. Presently, our results, which are based on probing the expression profiles of several immune response genes (Figure 4 and Figure S3), would seem to demand that the molecular mechanisms governing the time-of-day differences in survival for flies with functional clocks are different from those affecting survival rates in per^{01} and Clk^{Jrk} flies. Otherwise stated, it does not appear likely that the survival rates of per^{01} and Clk^{Jrk} flies are due simply to their clocks being pegged or held at a phase that is similar to either ZT/CT5 or ZT/CT17 in wild-type flies, respectively. Although future work will be required to resolve the molecular underpinnings governing the differential clock mutant effects on survival, these considerations suggest that core clock genes have "non-circadian" related roles (i.e., roles not solely limited to their functions in timekeeping) in fighting microbial infections. Indeed, our findings add to a growing list of physiological and behavioral pathways that are differentially regulated in different clock mutants; e.g., mutations in *per* but not *tim*, *Clk*, or *cyc* play a key role in long-term memory formation in *Drosophila* [19].

If the ability to evoke a stronger response at night enhances the efficacy of fighting a microbial infection, why restrict it to the night? It is widely thought that maintaining an optimal immune system is metabolically costly, competing for limited metabolic resources with other energetically demanding activities such as foraging or mating [20]. Within this framework we suggest that the clock might function as a temporal sieve to ensure the proper allocation of metabolic resources at biologically desirable times. From a more medical perspective, our results suggest that the innate immune system is a prime target for interventions based on chronobiological considerations in the hopes of boosting the ability to combat pathogenic infections.

Supplemental Data

Supplemental Experimental Procedures, four figures, and five tables are available at http://www.current-biology.com/cgi/content/full/18/3/195/DC1/.

Acknowledgments

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